

## SPECIFICATION

### ALPHA 1-6 FUCOSYLTRANSFERASE

#### Technical Field

INS A1

The present invention relates to an  $\alpha$ 1-6 fucosyltransferase derived from pig or human. More particularly, the present invention relates to a novel  $\alpha$ 1-6 fucosyltransferase derived from human, which is an enzyme that transfers fucose from guanosine diphosphate (GDP)-fucose by  $\alpha$ 1 $\rightarrow$ 6 linkage to N-acetylglucosamine (GlcNAc) bound to Asn at the stem of asparagine type sugar chain (Asn type sugar chain) and which is useful in the field of glyco-technology for modification and synthesis of sugar chain and/or for the diagnosis of diseases such as malignant tumor, and to a gene encoding said enzyme.

#### Background Art

The structure and function of sugar chain moiety of complex carbohydrates, such as glycoprotein and glycolipid, derived from higher organisms have been drawing much attention in recent years, and many studies are under way. While a sugar chain is formed by the action of glycohydrolase and glycosyltransferase, glycosyltransferase contributes greatly to its formation.

Using a sugar nucleotide as a sugar donor, glycosyltransferase transfers a sugar to a receptor sugar chain, thereby to elongate the sugar chain. The specificity for the structure of receptor sugar chain is stringent, such that one glycoside linkage is formed by the corresponding one transferase. Hence, glycosyltransferases are used for structural studies of sugar moiety of complex carbohydrate, for facilitated synthesis of a particular sugar chain structure, and for modification of native sugar chain structure.

Besides, glycosyltransferases are expected to be usable for the modification of the nature of complex carbohydrate and cells, by means of artificial alteration of sugar chain. For this end, the development of various glycosyltransferases having identified substrate specificity has been awaited.

An  $\alpha$ 1-6 fucosyltransferase is an important enzyme found in Golgi

apparatus of organelle, which is considered to be one of the enzymes that control processing of asparagine-linked sugar chain. Therefore, the enzyme will be useful for the elucidation of control mechanism and control of formation of sugar chain structure, once acted on an asparagine-linked sugar chain.

In addition, the activity of  $\alpha$ 1-6 fucosyltransferase and the proportion of reaction products of this enzyme are known to increase in certain diseases such as liver cancer and cystic fibrosis. Therefore, a rapid development of the method for diagnosis of these diseases has been desired, which involves determination of the activity of this enzyme, Northern blot using a cDNA encoding  $\alpha$ 1-6 fucosyltransferase, or RT-PCR assay of mRNA amount transcribed and expressed in the living body.

The activity of  $\alpha$ 1-6 fucosyltransferases has been detected in body fluids or organs of various animals and culture cells thereof, and there has been known, as a purified enzyme product, an enzyme derived from human cystic fibrosis cell homogenates [Journal of Biological Chemistry, vol. 266, pp. 21572-21577 (1991)]. According to this report, however, the enzyme is associated with drawbacks in that (1) its optimum pH is 5.6 which is different from physiological pH, (2) it has relatively low molecular weights (34,000 and 39,000) by SDS-polyacrylamide gel electrophoresis, (3) its large scale and stable supply is practically unattainable due to its being derived from human cell, and others.

This enzyme is obtained as a membrane-bound enzyme, and requires bovine serum for culturing the cells, which in turn results in difficult purification of the enzyme and a huge amount of money necessary for culture of the cells to be a starting material. Consequently, stable supply of this enzyme preparation is all but impractical.

While a chemical synthesis is often employed for synthesizing a sugar chain, the synthesis of oligosaccharides requires many steps that have been necessitated by its complicated synthesis route and specificity of the reaction, so that it involves various practical

problems. Particularly, binding of fucose to GlcNAc bound to Asn of asparagine-linked sugar chain by  $\alpha 1 \rightarrow 6$  linkage is extremely difficult due to the instability of fucose.

#### Disclosure of the Invention

It is therefore an object of the present invention to stably provide an  $\alpha 1-6$  fucosyltransferase in large amounts, which is useful as a reagent for structural analysis of sugar chain or glyco-technology, or as diagnostics.

Another object of the present invention is to provide a method of producing  $\alpha 1-6$  fucosyltransferase in large amounts by the use of a human- or porcine-derived  $\alpha 1-6$  fucosyltransferase gene. It is aimed to use such specific genes so as to enable development of a method for diagnosis of diseases by Northern blot using a DNA encoding said enzyme, or by RT-PCR assay of mRNA amount transcribed and expressed in the living body.

In an attempt to achieve the above-mentioned objects, the present inventors started the study of an enzyme capable of linking fucose to GlcNAc linked to Asn of asparagine type sugar chain by  $\alpha 1 \rightarrow 6$  linkage, using a fluorescence-labeled substrate analogous to an asparagine type sugar chain which is a receptor of this enzyme. As a result, they have found the activity of this enzyme in the extract fractions of porcine brain which is readily available as a starting material to be purified, and they have purified said enzyme from said fractions and elucidated the enzymatic and physico-chemical properties, which resulted in the completion of the invention.

Accordingly, the present invention relates to a porcine-derived  $\alpha 1-6$  fucosyltransferase having the following physico-chemical properties (hereinafter this enzyme is referred to as porcine  $\alpha 1-6$  fucosyltransferase).

(1) Action: transferring fucose from guanosine diphosphate-fucose to the hydroxy group at 6-position of GluNAc closest to R of a receptor  $(\text{GlcNAc}\beta 1-2\text{Man}\alpha 1-6)(\text{GlcNAc}\beta 1-2\text{Man}\alpha 1-3)\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc-R}$  wherein R is an asparagine residue or a peptide chain

carrying said residue, whereby to form (GlcNAc $\beta$ 1-2Man $\alpha$ 1-6)-(GlcNAc $\beta$ 1-2Man $\alpha$ 1-3)Man $\beta$ 1-4GlcNAc $\beta$ 1-4(Fuc $\alpha$ 1-6)GlucNAc-R.

In the above formula, asparagine residue at R is a residue wherein the acid amide group at the side chain of asparagine is bound to the hydroxy group at the anomer position of the reducing terminal of sugar chain, and a peptide chain having said residue is a peptide chain having said residue in the peptide to which two or more amino acids are bound, which is preferably a peptide chain having -Asn-(X)-Ser/Thr-.

(2) optimum pH : about 7.0

(3) pH stability : stable in the pH range of 4.0-10.0 by treatment at 4°C for 5 hours

(4) optimum temperature : about 30-37°C

(5) inhibition or activation : no requirement for divalent metal ion for expression of activity; no inhibition of activity even in the presence of 5 mM EDTA

(6) molecular weight : about 60,000 by SDS-polyacrylamide gel electrophoresis.

The present inventors have purified  $\alpha$ 1-6 fucosyltransferase alone from porcine brain, analyzed the amino acid sequence of this protein and cloned a gene based on the partial amino acid sequence to accomplish the present invention.

That is, the present invention provides a gene encoding porcine  $\alpha$ 1-6 fucosyltransferase.

The present invention also provides an expression vector containing a gene encoding porcine  $\alpha$ 1-6 fucosyltransferase.

The present invention further provides a transformant cell obtained by transforming a host cell with an expression vector containing a gene encoding porcine  $\alpha$ 1-6 fucosyltransferase.

The present invention yet provides a method for producing a recombinant  $\alpha$ 1-6 fucosyltransferase, comprising culturing a transformant cell obtained by transforming a host cell with an expression vector containing a gene encoding porcine  $\alpha$ 1-6 fucosyltransferase, and harvesting the  $\alpha$ 1-6 fucosyltransferase from the

culture thereof.

The present inventors have reached the present invention by purifying protein having an  $\alpha$ 1-6 fucosyltransferase activity from human cell culture broth and elucidating its enzymatic property.

Accordingly, the present invention relates to an  $\alpha$ 1-6 fucosyltransferase derived from human, having the following physico-chemical property (hereinafter this enzyme is to be referred to as human  $\alpha$ 1-6 fucosyltransferase).

(1) Action: transferring fucose from guanosine diphosphate-fucose to the hydroxy group at 6-position of GlcNAc closest to R of a receptor  $(\text{GlcNAc}\beta 1-2\text{Man}\alpha 1-6)(\text{GlcNAc}\beta 1-2\text{Man}\alpha 1-3)\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc}-\text{R}$  wherein R is an asparagine residue or a peptide chain carrying said residue, whereby to form  $(\text{GlcNAc}\beta 1-2\text{Man}\alpha 1-6)(\text{GlcNAc}\beta 1-2\text{Man}\alpha 1-3)\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4(\text{Fuc}\alpha 1-6)\text{GlcNAc}-\text{R}$ .

In the above formula, asparagine residue at R is a residue wherein the acid amide group at the side chain of asparagine is bound to the reducing terminal hydroxy group of sugar chain, and a peptide chain having said residue is a peptide chain having said residue in the peptide to which two or more amino acids are bound, which is preferably a peptide chain having -Asn-(X)-Ser/Thr-.

(2) optimum pH : about 7.5

(3) pH stability : stable in the pH range of 4.0-10.0 by treatment at 4°C for 5 hours

(4) optimum temperature : about 30-37°C

(5) inhibition or activation : no requirement for divalent metal ion for expression of activity; no inhibition of activity even in the presence of 5 mM EDTA

(6) molecular weight : about 60,000 by SDS-polyacrylamide gel electrophoresis.

The present inventors have purified  $\alpha$ 1-6 fucosyltransferase alone from human culture cell, analyzed the amino acid sequence of this protein and cloned a gene based on the partial amino acid sequence to accomplish the present invention.

That is, the present invention provides a gene encoding human  $\alpha 1-6$  fucosyltransferase.

The present invention also provides an expression vector containing a gene encoding human  $\alpha 1-6$  fucosyltransferase.

The present invention further provides a transformant cell obtained by transforming a host cell with an expression vector containing a gene encoding human  $\alpha 1-6$  fucosyltransferase.

The present invention yet provides a method for producing a recombinant  $\alpha 1-6$  fucosyltransferase, comprising culturing a transformant cell obtained by transforming a host cell with an expression vector containing a gene encoding human  $\alpha 1-6$  fucosyltransferase, and harvesting the  $\alpha 1-6$  fucosyltransferase from the culture thereof.

The starting material for the purification of the enzyme of the present invention is, for example, the organ and body fluid of pig having  $\alpha 1-6$  fucosyltransferase activity. Examples of the organ include brain, spermary, pancreas, lung, kidney and the like. The body fluid of pig such as blood and sera can be also used.

The porcine  $\alpha 1-6$  fucosyltransferase of the present invention can be obtained by preparing a crude extract containing the enzyme from, for example, homogenates of porcine brain and separating the enzyme from this extract. In this case, since  $\alpha 1-6$  fucosyltransferase in the porcine brain is a membrane-bound enzyme, a crude extract solution containing the enzyme is generally obtained from brain lysate using a suitable surfactant. This extract undergoes various known purification steps to give a purified enzyme product. The purification may include, for example, concentration using an ultrafiltration membrane, desalting, affinity column chromatography wherein a substrate analog is immobilized, ion exchange column chromatography, hydrophobic column chromatography and the like in suitable combination to give a substantially homogeneous enzyme product which is free of contaminant proteins such as other transferases. For example, porcine brain is disrupted in a Waring blender in a phosphate buffer and membrane



MES buffer containing 62.5  $\mu$ M of fluorescence-labeled receptor substrate of the above formula and 625  $\mu$ M of a donor substrate (GDP-fucose), pH 7.0, 40  $\mu$ l, and mixed. The mixture is reacted at 37°C for one hour, and boiled for 5 minutes to stop the reaction. The reaction mixture is subjected to high performance liquid chromatography and the peak of the reaction product is assayed with a fluorescence detector. One unit of the enzyme amount corresponds to the amount capable of forming 1 pmole of GlcNAc  $\beta$ 1-2Man $\alpha$ 1-6(GlcNAc $\beta$ 1-2Man $\alpha$ 1-3)Man $\beta$ 1-4GlcNAc $\beta$ 1-4(Fuc $\alpha$ 1-6)GlcNAc-R (wherein R is Asn-NH-(CH<sub>2</sub>)<sub>4</sub>-NH-pyridine) in one minute under these conditions.

(3) Optimum pH:

The  $\alpha$ 1-6 fucosyltransferase derived from porcine brain (hereinafter this enzyme is referred to as porcine brain  $\alpha$ 1-6 fucosyltransferase) shows a high activity at nearly pH 7.0-7.5.

(4) pH Stability:

The porcine brain  $\alpha$ 1-6 fucosyltransferase is relatively stable at pH 4-10, and more stable at pH 5-9.

(5) Optimum temperature:

The porcine brain  $\alpha$ 1-6 fucosyltransferase has an optimum temperature at nearly 37°C and retains sufficient activity at 20-40°C.

(6) Divalent metal ion requirement:

The porcine brain  $\alpha$ 1-6 fucosyltransferase shows sufficient activity even in the absence of divalent metal ion, such as magnesium, manganese and the like. It also shows sufficient activity even in the presence of 5 mM EDTA, which is a chelating agent.

(7) Molecular weight:

A purified product of the porcine brain  $\alpha$ 1-6 fucosyltransferase shows a single band at a molecular weight of about 60,000 by SDS-polyacrylamide gel electrophoresis.

Judging from the above properties, the porcine brain  $\alpha$ 1-6 fucosyltransferase is a novel enzyme apparently different from conventionally known  $\alpha$ 1-6 fucosyltransferase derived from human cystic fibrosis cells (optimum pH 5.6, molecular weights 34,000 and 39,000),



in terms of optimum pH, metal ion requirement and molecular weight.

The inventive porcine  $\alpha$ 1-6 fucosyltransferase is expected to be extremely useful for (1) synthesis of sugar chain compounds wherein sugar chain compounds having 1-6 fucose are synthesized using the enzyme of the present invention, (2) modification of sugar chain structure and functional analysis wherein a fucose is newly introduced into asparagine type sugar chain to artificially modify the sugar chain structure, whereby changes in cell function and control mechanism of the processing of complex carbohydrate, as well as the role of sugar chain, can be elucidated, (3) diagnosis of lesions based on enzyme activity wherein diseases such as cancer can be diagnosed by determining the activity of the enzyme of the present invention which reflects various lesions caused by tumorigenic transformation, (4) diagnosis of various diseases wherein a specific antibody against the enzyme of the present invention is prepared and used for the diagnosis, and the like.

Another aspect of the present invention is a gene encoding porcine  $\alpha$ 1-6 fucosyltransferase, which includes a gene encoding  $\alpha$ 1-6 fucosyltransferase and including a gene encoding amino acid sequence depicted in Sequence Listing, SEQ ID NO:2. A different embodiment thereof is a gene encoding  $\alpha$ 1-6 fucosyltransferase and including nucleotide sequence depicted in Sequence Listing, SEQ ID NO:1.

One aspect of the present invention is a gene encoding porcine  $\alpha$ 1-6 fucosyltransferase and including a gene encoding an amino acid sequence resulting from substitution, insertion, deletion or addition with respect to at least one amino acid of the amino acid sequence depicted in Sequence Listing, SEQ ID NO:2.

Another aspect of the present invention is a gene encoding porcine  $\alpha$ 1-6 fucosyltransferase and including a nucleotide sequence resulting from substitution, insertion, deletion or addition with respect to at least one nucleotide of the nucleotide sequence depicted in Sequence Listing, SEQ ID NO:1.

The present invention also includes, as one aspect thereof, a gene that hybridizes to at least a part of a gene encoding porcine  $\alpha$ 1-6

fucosyltransferase and including nucleotide sequence depicted in Sequence Listing, SEQ ID NO:1.

The expression vector of the present invention contains a gene encoding the above-mentioned porcine  $\alpha$ 1-6 fucosyltransferase.

The transformant host cell of the present invention has been transformed with the above-mentioned expression vector.

The host cell is exemplified by microorganisms, such as *Escherichia coli*, yeast, bacterial cells and the like. It also includes animal cells such as insect cells, COS-1 cells, CHO cells and the like, and plant cells, such as tobacco cells, *Arabidopsis* cells and the like.

The vector may be any which is selected according to the host to be transformed. In the case of *Escherichia coli*, for example, pUC19 may be used; in the case of yeast, pYEUra3<sup>TM</sup> may be used; in the case of insect cells, pBLUE Bac4 may be used; in the case of COS-1 cells, pSVK3 may be used; and in the case of tobacco cells and *Arabidopsis* cells, pBI may be used.

The method for preparing the inventive recombinant  $\alpha$ 1-6 fucosyltransferase includes culturing the above-mentioned transformant cells and harvesting  $\alpha$ 1-6 fucosyltransferase from the culture.

According to the present invention,  $\alpha$ 1-6 fucosyltransferase alone is purified from porcine brain, and subjected to amino acid analysis of this protein. Its partial amino acid sequence is determined and a primer for PCR is prepared based on the amino acid sequence. Using this primer, PCR is performed using cDNAs derived from porcine brain as a template to amplify a gene encoding  $\alpha$ 1-6 fucosyltransferase to give a probe. This probe is used to screen clones containing cDNA encoding  $\alpha$ 1-6 fucosyltransferase, from the cDNA library derived from porcine brain. The cDNA encoding  $\alpha$ 1-6 fucosyltransferase is isolated and used to express  $\alpha$ 1-6 fucosyltransferase.

To be specific, the purified porcine  $\alpha$ 1-6 fucosyltransferase is used to analyze amino acid sequences. For example, SDS-polyacrylamide gel electrophoresis is applied, after which the protein is transferred to PVDF membrane by electroblotting, and the PVDF membrane containing

ca. 60 kDa band is cut out and subjected to sequencing using a protein sequencer. As a result, the amino acid sequence of the amino terminal of  $\alpha$ 1-6 fucosyltransferase depicted in Sequence Listing, SEQ ID NO:3 is obtained.

Separately, purified  $\alpha$ 1-6 fucosyltransferase is subjected to SDS-polyacrylamide gel electrophoresis and the peptide fragments separated by electrophoresis are transferred to PVDF membrane by electroblotting. Then, the PVDF membrane containing 60 kDa band is cut out and lysed on said PVDF membrane, using, for example, a protease such as lysylendopeptidase. The lysate is extracted from the sections of said PVDF membrane, and the extract is subjected to reversed phase high performance liquid chromatography to separate the lysate.

Then, using the amino acid sequences, a mixed primer for PCR is prepared. For example, a mixed primer having a nucleotide sequence depicted in SEQ ID NO:7 is synthesized from the amino acid sequence depicted in SEQ ID NO:3, and a mixed primer having a nucleotide sequence depicted in SEQ ID NO:8 is synthesized from the amino acid sequence depicted in SEQ ID NO:4, respectively using a DNA synthesizer, and used for the screening of cDNA of  $\alpha$ 1-6 fucosyltransferase.

For example, 25 cycles of PCR are performed to amplify DNA fragments of ca. 1.45 kbp, using cDNA from porcine brain as a template and mixed primers of SEQ ID NO:7 and SEQ ID NO:8, wherein PCR at 94°C (1 min), 55°C (2 min) and 72°C (3 min) is one cycle.

Then, using the amplified DNA fragments as a probe, clones containing cDNA encoding  $\alpha$ 1-6 fucosyltransferase are screened from the cDNA library derived from porcine brain by a plaque hybridization method. The cDNA encoding  $\alpha$ 1-6 fucosyltransferase can be isolated from the obtained clones. The nucleotide sequence of the obtained cDNA and the amino acid sequence deduced from said nucleotide sequence are shown in SEQ ID NO:1 and SEQ ID NO:2.

Said cDNA is subcloned into an expression vector such as pSVK3. The host cells, such as COS-1 cells, transformed with said subcloned plasmid, are cultured and  $\alpha$ 1-6 fucosyltransferase is obtained from the

culture.

In the present invention, the above-mentioned transformant cells are cultured and  $\alpha$ 1-6 fucosyltransferase is harvested from the culture, whereby recombinant  $\alpha$ 1-6 fucosyltransferase is obtained. The method for harvesting the enzyme from the culture is a conventional one.

The gene encoding the porcine  $\alpha$ 1-6 fucosyltransferase of the present invention and DNA fragments (which are the lysates thereof) may be used for the detection of the expression of  $\alpha$ 1-6 fucosyltransferase in the living body, and thus are useful for the genetic diagnosis of certain diseases such as liver cancer and cystic fibrosis.

In addition, the polypeptide that is encoded by these genes can be used to immunologically prepare various antibodies which are useful for diagnosis and purification of  $\alpha$ 1-6 fucosyltransferase.

The starting material for the purification of the enzyme in this invention may be any as long as it is a human cell culture medium exhibiting  $\alpha$ 1-6 fucosyltransferase activity. For example, human pancreatic cancer cells, human gastric cancer cells, human myeloma tumor cells and the like may be used as the cells having  $\alpha$ 1-6 fucosyltransferase activity.

While the human  $\alpha$ 1-6 fucosyltransferase is present in the cell membrane as a membrane-bound enzyme, it is cleaved by protease at a site unaffecting the enzyme activity and released into the culture medium as a soluble enzyme. Thus, the culture medium can be used as a crude enzyme solution, without complicated steps such as disruption of cells and solubilizing of the enzyme. Besides, the use of cells capable of growth in serum-free media enables economical production of a crude enzyme solution having a high purity. The culture medium is concentrated and desalted, and subjected to ion exchange chromatography, affinity chromatography and the like to give a purified enzyme product free of contaminant transferases and glycosidase activity.

$\alpha$ 1-6 Fucosyltransferase is purified from human gastric cancer cells by, for example, culturing human gastric cancer cell MKN45 without

serum and purifying the enzyme from the obtained culture medium. In this case,  $\alpha$ 1-6 fucosyltransferase of human gastric cancer cell MKN45 is cleaved by protease in the cells at a site unaffecting the enzyme activity and released into culture medium as a soluble  $\alpha$ 1-6 fucosyltransferase. Therefore, the culture medium can be used as a crude enzyme solution, without complicated steps such as disruption of cells and solubilizing of the enzyme with a surfactant. The crude enzyme solution is subjected to known purification steps to give a purified enzyme product.

In the present invention, a serum-free culture medium of human gastric cancer cell MKN45 is concentrated by filtration through an ultrafiltration membrane, and then the buffer is changed to a Tris-HCl buffer containing 5 mM 2-mercaptoethanol and 0.1% CHAPS [3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate], pH 7.5, to give a crude enzyme solution.

This enzyme solution is subjected to column chromatography using Q-sepharose, GDP-hexanolamine-sepharose, (GlcNAc $\beta$ 1-2Man $\alpha$ 1-6)(GlcNAc $\beta$ 1-2Man $\alpha$ 1-3)Man $\beta$ 1-4GlcNAc $\beta$ 1-4GlcNAc-asparagine-sepharose and the like to collect active fractions, from which the fucosyltransferase of the present invention can be purified.

The physico-chemical property of human  $\alpha$ 1-6 fucosyltransferase of the present invention is as follows.

(1) Action: transferring fucose from guanosine diphosphate-fucose to the hydroxy group at 6-position of GlcNAc closest to R of a receptor (GlcNAc $\beta$ 1-2Man $\alpha$ 1-6)(GlcNAc $\beta$ 1-2Man $\alpha$ 1-3)Man $\beta$ 1-4GlcNAc $\beta$ 1-4GlcNAc-R wherein R is an asparagine residue or a peptide chain carrying said residue, whereby to form (GlcNAc $\beta$ 1-2Man $\alpha$ 1-6)-(GlcNAc $\beta$ 1-2Man $\alpha$ 1-3)Man $\beta$ 1-4GlcNAc $\beta$ 1-4(Fuc $\alpha$ 1-6)GlcNAc-R.

(2) Determination of enzyme activity:

The activity of the human  $\alpha$ 1-6 fucosyltransferase was determined as follows. That is, a compound of the above-mentioned formula, wherein the asparagine on the end of sugar chain was fluorescence-labeled with 4-(2-pyridylamino)butylamine [PABA:  $-\text{NH}_2(\text{CH}_2)_4-\text{NH}-$

pyridine], was used as a substrate for determination of enzyme activity. By the use of this substrate, the product from the enzyme reaction, wherein fucose is transferred by  $\alpha 1 \rightarrow 6$  linkage, can be assayed by detecting fluorescence by high performance liquid chromatography.

Specifically, the determination included the following steps. An enzyme solution (10  $\mu$ l) was added to a 250 mM MES buffer containing 62.5  $\mu$ M of fluorescence-labeled receptor substrate of the above formula and 625  $\mu$ M of a donor substrate (GDP-fucose), pH 7.0, 40  $\mu$ l, and mixed. The mixture was reacted at 37°C for one hour, and boiled for 5 minutes to stop the reaction. The reaction mixture is subjected to high performance liquid chromatography and the peak of the reaction product is assayed with a fluorescence detector.

One unit of the enzyme amount corresponded to the amount capable of producing 1 pmole of  $\text{GlcNAc}\beta 1\text{-2Man}\alpha 1\text{-6(GlcNAc}\beta 1\text{-2Man}\alpha 1\text{-3)-Man}\beta 1\text{-4GlcNAc}\beta 1\text{-4(Fuc}\alpha 1\text{-6)GlcNAc-R}$  (wherein R is  $\text{Asn-NH-(CH}_2\text{)}_4\text{-NH-Pyridine}$ ) in one minute under these conditions.

(3) Optimum pH:

The human  $\alpha 1\text{-6}$  fucosyltransferase shows high activity at nearly pH 7.0-7.5, as shown by a curve in Fig. 1. In Fig. 1, the determination was performed using 500 mM MES buffer (black circle) at pH 4.5-7.5 and 100 mM Tris-HCl buffer (white circle) at pH 7.0-9.0.

(4) pH Stability:

The human  $\alpha 1\text{-6}$  fucosyltransferase is stable at about pH 4-10, particularly at pH 5-9, as shown in Fig. 2. The buffers used for the determination were 50 mM acetate buffer (black triangle) at pH 3.5-5.5, 50 mM MES buffer (black circle) at pH 5.5-7.5, 50 mM Tris-HCl buffer (white circle) at pH 7.5-9.0, and sodium hydrogencarbonate buffer (white triangle) at pH 9.0-11.5. The enzyme of the present invention was treated in each buffer at each pH at 4°C for 5 hours, and the residual activity was determined. Fig. 1 is a graph showing the relationship between pH (axis of abscissa) and relative activity (% axis of ordinate) of the human  $\alpha 1\text{-6}$  fucosyltransferase obtained by the

present invention, and Fig. 2 is a graph showing pH (axis of abscissa) and residual activity (% , axis of ordinate).

(5) Optimum temperature:

The human  $\alpha$ 1-6 fucosyltransferase has an optimum temperature at nearly 37°C as shown in Fig. 3 and is usable at 20-40°C. A frozen product thereof is stable at -20°C for at least several months.

(6) Divalent metal ion requirement:

Many glycosyltransferases require divalent metal ion for their activity, such as magnesium, manganese and the like. This human  $\alpha$ 1-6 fucosyltransferase shows sufficient activity in the absence of divalent metal ion or in the presence of EDTA, which is a chelating agent, and does not require divalent metal ion.

(7) Molecular weight:

A purified product of the human  $\alpha$ 1-6 fucosyltransferase of the present invention shows a single band at a molecular weight of about 60,000 by SDS-polyacrylamide gel electrophoresis.

(8) Morphology:

While the human  $\alpha$ 1-6 fucosyltransferase is intrinsically present in cell membrane as a membrane-bound enzyme, it is cleaved by protease in the cultured cell at a site unaffected the enzyme activity and released into a culture medium as a soluble enzyme permitting easy handling, unlike porcine-derived  $\alpha$ 1-6 fucosyltransferase and  $\alpha$ 1-6 fucosyltransferase derived from human cystic fibrosis cells heretofore reported.

Judging from the above properties, the human  $\alpha$ 1-6 fucosyltransferase is a novel enzyme apparently different from conventionally known  $\alpha$ 1-6 fucosyltransferase derived from human cystic fibrosis cells (optimum pH 6.5, molecular weights 34,000 and 39,000), in terms of optimum pH, metal requirement and molecular weight.

The human  $\alpha$ 1-6 fucosyltransferase is used for the following purposes.

(1) Artificial modification of sugar chain structure by introducing fucose anew into the asparagine-linked sugar chain, whereby cell

apparatus and control mechanism of processing of sugar chain of complex carbohydrate, as well as the role of sugar chain, can be elucidated.

(2) Diagnosis of various diseases based on the activity of the inventive enzyme.

(3) Diagnosis of various diseases wherein a specific antibody against the enzyme of the present invention is prepared and used for the diagnosis.

SEA 11  
The present invention is a gene encoding human  $\alpha$ 1-6 fucosyltransferase, which includes, as one embodiment, a gene encoding  $\alpha$ 1-6 fucosyltransferase and including a gene encoding an amino acid sequence depicted in Sequence Listing, SEQ ID NO:10. A different embodiment thereof is a gene encoding  $\alpha$ 1-6 fucosyltransferase inclusive of nucleotide sequence depicted in Sequence Listing, SEQ ID NO:9. A further aspect of the present invention is a gene encoding  $\alpha$ 1-6 fucosyltransferase and including a nucleotide sequence from 198th adenine to 1919th guanine as depicted in Sequence Listing, SEQ ID NO:9.

One aspect of the present invention is a gene encoding  $\alpha$ 1-6 fucosyltransferase and including a gene encoding an amino acid sequence resulting from substitution, insertion, deletion or addition with respect to at least one amino acid of the amino acid sequence depicted in Sequence Listing, SEQ ID NO:10.

Another aspect of the present invention is a gene encoding  $\alpha$ 1-6 fucosyltransferase and including a nucleotide sequence resulting from substitution, insertion, deletion or addition with respect to at least one nucleotide of the nucleotide sequence depicted in Sequence Listing, SEQ ID NO:9.

The present invention also includes, as one embodiment, a gene which hybridizes to at least a part of gene encoding  $\alpha$ 1-6 fucosyltransferase and including nucleotide sequence depicted in Sequence Listing, SEQ ID NO:9.

The expression vector of the present invention contains a gene encoding the above-mentioned  $\alpha$ 1-6 fucosyltransferase.

The transformant host cell of the present invention has been



transformed with the above-mentioned expression vector.

The host cell is exemplified by microorganisms, such as *Escherichia coli*, yeast, bacterial cells and the like. It also includes animal cells such as insect cells, COS-1 cells, CHO cells and the like, and plant cells, such as tobacco cells, *Arabidopsis* cells and the like.

The vector may be any which is selected according to the host to be transformed. In the case of *Escherichia coli*, for example, pUC19 may be used; in the case of yeast, pYEura3<sup>TM</sup> may be used; in the case of insect cells, pBLUE Bac4 may be used; in the case of COS-1 cells, pSVK3 may be used; and in the case of tobacco cells and *Arabidopsis* cells, pBI may be used.

The method for preparing the recombinant  $\alpha$ 1-6 fucosyltransferase includes culturing the above-mentioned transformant cells and harvesting  $\alpha$ 1-6 fucosyltransferase from the culture.

According to the present invention,  $\alpha$ 1-6 fucosyltransferase alone is purified from human gastric cancer cells, and subjected to amino acid analysis of this protein. Its partial amino acid sequence is determined and a primer for PCR is prepared based on the amino acid sequence. Using this primer, PCR is performed using cDNAs derived from human gastric cancer cells as a template to amplify a gene encoding  $\alpha$ 1-6 fucosyltransferase to give a probe. This probe is used to screen clones containing cDNA encoding  $\alpha$ 1-6 fucosyltransferase, from the cDNA library derived from human gastric cancer cells. The cDNA encoding  $\alpha$ 1-6 fucosyltransferase is isolated and used to express  $\alpha$ 1-6 fucosyltransferase.

To be specific, the purified  $\alpha$ 1-6 fucosyltransferase is used to analyze amino acid sequence. For example, it is subjected to SDS-polyacrylamide gel electrophoresis, after which the protein is transferred to PVDF membrane by electroblotting, and the PVDF membrane containing ca. 60 kDa band is cut out and subjected to sequencing by a protein sequencer. As a result, the amino acid sequence of the amino terminal of  $\alpha$ 1-6 fucosyltransferase depicted in Sequence Listing, SEQ ID NO:11 is obtained.

Separately, purified  $\alpha$ 1-6 fucosyltransferase is subjected to SDS-polyacrylamide gel electrophoresis, along with a protease such as lysylendopeptidase, and the peptide fragments separated by electrophoresis are transferred to PVDF membrane by electroblotting. Then, the band containing the peptide fragments is cut out and subjected to sequencing with a protein sequencer. Thus, partial amino acid sequences of  $\alpha$ 1-6 fucosyltransferase as depicted in Sequence Listing, SEQ ID NO:12 and SEQ ID NO:13 are obtained. Then, using these amino acid sequences, a mixed primer for PCR is prepared. For example, a mixed primer having a nucleotide sequence depicted in SEQ ID NO:14 is synthesized from the amino acid sequence depicted in SEQ ID NO:12, and a mixed primer having a nucleotide sequence depicted in SEQ ID NO:15 is synthesized from the amino acid sequence depicted in SEQ ID NO:13, respectively using a DNA synthesizer, and used for the screening of cDNA of  $\alpha$ 1-6 fucosyltransferase.

For example, 36 cycles of PCR are performed to amplify the DNA fragments of ca. 200 bp, using cDNA from human gastric cancer cells as a template and mixed primers of SEQ ID NO:14 and SEQ ID NO:15, wherein PCR at 94°C (30 sec), 46°C (30 sec) and 72°C (1.5 min) is one cycle.

Then, using the amplified DNA fragments as a probe, clones containing cDNA encoding  $\alpha$ 1-6 fucosyltransferase are screened from the cDNA library derived from human gastric cancer cells by a plaque hybridization method. The cDNA encoding  $\alpha$ 1-6 fucosyltransferase can be isolated from the obtained clones. The nucleotide sequence of the obtained cDNA and the amino acid sequences deduced from said nucleotide sequence are shown in SEQ ID NO:9 and SEQ ID NO:10.

Said cDNA is subcloned into an expression vector such as pSVK3. The host cells such as COS-1 cells transformed with said subcloned plasmid are cultured and  $\alpha$ 1-6 fucosyltransferase is obtained from the culture.

In the present invention, the above-mentioned transformant cells are cultured and  $\alpha$ 1-6 fucosyltransferase is harvested from the culture, whereby a recombinant  $\alpha$ 1-6 fucosyltransferase is obtained.

The method for harvesting the enzyme from the culture is a conventional one.

The gene encoding the human  $\alpha$ 1-6 fucosyltransferase of the present invention and DNA fragments (which are the lysates thereof) may be used for the determination of the expression of  $\alpha$ 1-6 fucosyltransferase in the living body and thus are useful for genetic diagnosis of certain diseases such as liver cancer and cystic fibrosis.

In addition, the polypeptide that is encoded by these genes can be used to immunologically prepare various antibodies which are useful for diagnosis and purification of  $\alpha$ 1-6 fucosyltransferase.

#### Brief Description of the Drawings

Fig. 1 shows optimum pH of the porcine brain  $\alpha$ 1-6 fucosyltransferase of the present invention.

Fig. 2 shows pH stability of the porcine brain  $\alpha$ 1-6 fucosyltransferase of the present invention.

Fig. 3 shows optimum temperature of the porcine brain  $\alpha$ 1-6 fucosyltransferase of the present invention.

Fig. 4 shows optimum pH of the human  $\alpha$ 1-6 fucosyltransferase of the present invention.

Fig. 5 shows pH stability of the human  $\alpha$ 1-6 fucosyltransferase of the present invention.

Fig. 6 shows optimum temperature of the human  $\alpha$ 1-6 fucosyltransferase of the present invention.

#### Embodiment of the Invention

The present invention is described in more detail by way of Examples.

In the present invention, the enzyme activity is determined as follows.

A compound of the following formula, wherein the asparagine on the end of sugar chain had been fluorescence-labeled with 4-(2-pyridyl-amino)butylamine [PABA:  $-\text{NH}(\text{CH}_2)_4-\text{NH}$ -pyridine] was used as a substrate for the determination of enzyme activity.

By the use of this substrate, the product from the enzyme reaction wherein fucose has been transferred by  $\alpha 1 \rightarrow 6$  linkage can be assayed by detecting the fluorescence by high performance liquid chromatography.

Specifically, the determination includes the following steps. A sample solution (10  $\mu$ l) and 1.25% Triton X-100 are added to a 250 mM MES buffer containing 62.5  $\mu$ M of fluorescence-labeled receptor substrate of the above formula and 625  $\mu$ M of a donor substrate (GDP-fucose), pH 7.0, 40  $\mu$ l, and mixed. The mixture is reacted at 37°C for one hour, and boiled for 5 minutes to stop the reaction. The reaction mixture is subjected to high performance liquid chromatography and the peak of the reaction product is assayed with a fluorescence detector. One unit of the enzyme amount corresponds to the amount capable of producing 1 pmole of  $\text{GlcNAc}\beta 1-2\text{Man}\alpha 1-6(\text{GlcNAc}\beta 1-2\text{Man}\alpha 1-3)-\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4(\text{Fuc}\alpha 1-6)\text{GlcNAc-R}$  (wherein R is  $\text{Asn-NH}-(\text{CH}_2)_4-\text{NH-pyridine}$ ) in one minute under these conditions.

#### Example 1

##### (1) Preparation of porcine brain lysate and crude extract solution

Porcine brain (100 g) was disrupted in a Waring blender in a 20 mM potassium phosphate buffer (pH 7.0) and membrane fractions were collected by ultracentrifugation. The membrane fractions were extracted with the same buffer containing Triton X-100 (concentration 0.5%) to extract the enzyme. After the extraction, the supernatants were collected by centrifugation to give an extract containing a crude enzyme.

##### (2) Purification of enzyme from crude extract solution

A  $\text{GlcNAc}\beta 1-2\text{Man}\alpha 1-6(\text{GlcNAc}\beta 1-2\text{Man}\alpha 1-3)\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc-asparagine-sepharose}$  column (column of asialoagalactoglycopeptide derived from transferrin) was equilibrated with a 20 mM

potassium phosphate buffer (pH 7.0) containing 0.05% Triton X-100 and 50 mM KCl, and the crude extract solution prepared in (1) above was applied. The column was washed with said buffer until the protein was not detected in the unadsorbed fractions. The active fractions were eluted with the same buffer containing 1M KCl. Then, the active fractions of the enzyme were concentrated using an ultrafiltration membrane and desalted, and applied to a GDP-hexanolamine-sepharose column equilibrated with the same buffer. The elution was performed using the same buffer containing 100 mM GDP. Then, the active fractions were collected and concentrated using an ultrafiltration membrane, and desalted to give porcine brain  $\alpha$ 1-6 fucosyltransferase. The porcine brain  $\alpha$ 1-6 fucosyltransferase thus obtained showed a single band at a molecular weight of about 60,000 by SDS-polyacrylamide gel electrophoresis. No other bands ascribed to impurities were found and the enzyme was free of other transferase activities, thus indicating that the enzyme obtained was highly purified.

The optimum pH (determined by changing the pH of buffer) of the enzyme of the present invention is shown in Fig. 1. The enzyme showed high activity at around pH 7.0-7.5. The buffer used was 200 mM MES buffer (black circle). In this graph, the axis of abscissa shows pH of  $\alpha$ 1-6 fucosyltransferase obtained in the present invention and the axis of ordinate shows relative activity (%).

The pH stability of the enzyme of the present invention was examined in the same manner. Fig. 2 shows residual activity after treating the enzyme in each buffer at each pH, 4°C for 5 hours. The enzyme was comparatively stable at about pH 4-10, and particularly stable at pH 5-9. The buffers used were 50 mM acetate buffer (black triangle) at pH 3.5-5.5, 50 mM MES buffer (black circle) at pH 5.5-7.5, 50 mM Tris-HCl buffer (white circle) at pH 7.5-9.0, and sodium hydrogencarbonate buffer (white triangle) at pH 9.0-11.5. The axis of abscissa of the graph shows pH of  $\alpha$ 1-6 fucosyltransferase obtained in the present invention and the axis of ordinate shows residual activity (%).

As shown in Fig. 3, the optimum temperature of the enzyme of the present invention was found to be at about 37°C and the enzyme was considered to retain sufficient activity in the range of 20-40°C. A frozen product thereof was stable at -20°C for at least several months. The buffer used was 200 mM MES buffer (black circle), pH 7.0. The axis of abscissa of the graph shows treatment temperature (°C) and the axis of ordinate shows relative activity (%) of the  $\alpha$ 1-6 fucosyltransferase obtained in the present invention.

While many glycosyltransferases require divalent metal ion for their activity, such as magnesium, manganese and the like, the enzyme showed sufficient activity in the absence of such divalent metal ion. Inasmuch as it showed sufficient activity even in the presence of 5 mM EDTA, which is a chelating agent, it is concluded that the enzyme does not require a divalent metal ion.

#### Example 2

Determination of amino terminal amino acid sequence of porcine brain  $\alpha$ 1-6 fucosyltransferase

Purified porcine brain  $\alpha$ 1-6 fucosyltransferase (5  $\mu$ g) was subjected to SDS-polyacrylamide gel electrophoresis, after which the protein was transferred to PVDF membrane (Millipore) by electroblotting. The PVDF membrane was stained with Coomassie Brilliant Blue G250, and a single band of porcine brain  $\alpha$ 1-6 fucosyltransferase was detected at 60 kDa.

Then, the PVDF membrane containing said band was cut out, and, after destaining with 50% methanol, subjected to Biosystem 473A protein sequencer (Applied Biosystems) to determine amino terminal amino acid sequence of  $\alpha$ 1-6 fucosyltransferase. The amino acid sequence determined is depicted in Sequence Listing, SEQ ID NO:3.

#### Example 3

Determination of partial amino acid sequence of porcine brain  $\alpha$ 1-6 fucosyltransferase

Purified porcine brain  $\alpha$ 1-6 fucosyltransferase (13  $\mu$ g) was subjected to SDS-polyacrylamide gel electrophoresis, after which the

protein was transferred to PVDF membrane (Millipore) by electroblotting. The PVDF membrane was stained with Coomassie Brilliant Blue G250, and a single band of porcine brain  $\alpha$ 1-6 fucosyltransferase was detected at 60 kDa.

Then, the PVDF membrane containing said band was cut out and destained with 50% methanol. Said PVDF membrane section was treated in 100 mM Tris-HCl buffer-5% acetonitrile (pH 8.2) containing 1  $\mu$ g of lysylendopeptidase, at 37°C for 12 hours for proteolysis. The PVDF membrane section which underwent proteolysis was ultrasonicated to extract the proteolysis product. The proteolysis product thus obtained was separated by a reversed phase high performance liquid chromatography using a C-18 column to give 3 peptide fragments. The substance containing said peptide fragments, which was separated by the reversed phase high performance liquid chromatography, was applied to polybrene-coated precycled glass fiber filter activated with trifluoroacetate and dried, and then subjected to Biosystem 473A protein sequencer (Applied Biosystems) to determine partial amino acid sequence of porcine brain  $\alpha$ 1-6 fucosyltransferase. The determined amino acid sequence is depicted in Sequence Listing, SEQ ID NOs:4-6.

#### Example 4

##### Preparation of probe DNA by PCR

Mixed primers shown in SEQ ID NO:7 and SEQ ID NO:8 were synthesized from the amino acid sequences obtained in Examples 2 and 3. The mixed primer shown in SEQ ID NO:7 was used as a sense primer, and the mixed primer shown in SEQ ID NO:8 was used as an antisense primer for PCR. To be specific, 25 cycles of PCR were performed wherein PCR at 94°C (1 min), 55°C (2 min) and 72°C (3 min) using 2  $\mu$ g of porcine brain-derived cDNA, 25 pmole of sense primer (mixed primer shown in SEQ ID NO:7), 25 pmole of antisense primer (mixed primer shown in SEQ ID NO:8) and a reaction mixture (50  $\mu$ l) of 50 mM potassium chloride-10 mM Tris-HCl buffer (pH 8.3)-1.5 mM magnesium chloride-0.001% gelatin-200  $\mu$ M dNTP, containing 2.5 units of Taq DNA polymerase was one cycle.

The reaction mixture (10  $\mu$ l) after PCR was subjected to 0.7%

agarose gel electrophoresis to confirm the PCR reaction product DNA fragments. As a result of PCR performed using a mixed primer shown in SEQ ID NO:7 and a mixed primer shown in SEQ ID NO:8 in combination, a 1.45 kbp DNA fragment was confirmed by agarose gel electrophoresis.

This DNA fragment was subcloned into plasmid pT7BLUEt-Vector (Novagen) and nucleotide sequence was confirmed. As a result, a DNA corresponding to the amino acid sequence depicted in Sequence Listing, SEQ ID NOs:3-6 was detected, whereby the DNA fragment was confirmed to be a part of  $\alpha$ 1-6 fucosyltransferase gene.

#### Example 5

Isolation of porcine brain  $\alpha$ 1-6 fucosyltransferase gene

The DNA fragments obtained in Example 4 were labeled with  $\alpha$ -<sup>32</sup>P dCTP (3000 Ci/mmol, Amersham) and used as a probe to screen clones containing cDNA encoding  $\alpha$ 1-6 fucosyltransferase, from porcine brain-derived  $\lambda$ gt11 cDNA library (Clonetech) by plaque hybridization method.

As a result of screening of about 400,000 plaques, 5 positive clones c1, c2, c3, c4 and c5 were obtained. Said clones c1 and c2 were postulated to contain a full length  $\alpha$ 1-6 fucosyltransferase gene in view of their length. Thus, the nucleotide sequences of c1 and c2 were determined, and a nucleotide sequence depicted in SEQ ID NO:1 was obtained.

#### Example 6

Expression of porcine brain  $\alpha$ 1-6 fucosyltransferase gene

The coding region of  $\alpha$ 1-6 fucosyltransferase gene was subcloned into expression vector pSVK3 from clones containing cDNA encoding porcine brain  $\alpha$ 1-6 fucosyltransferase obtained in Example 5. The expression vector containing said  $\alpha$ 1-6 fucosyltransferase gene was introduced into COS-1 cells. After 48 hours of incubation, culture cells were collected and the cells were disrupted. The enzyme activity of  $\alpha$ 1-6 fucosyltransferase in the obtained lysate was determined.

As a control, the enzyme activity of  $\alpha$ 1-6 fucosyltransferase in the lysate of COS-1 cells, into which mock pSVK3 had been introduced,



was determined. As a result, the control hardly showed activity, whereas COS-cells into which the expression vector containing said  $\alpha$ 1-6 fucosyltransferase gene had been introduced, showed a high activity of 2360 nmole/h/mg protein.

#### Example 7

(1) Preparation of crude enzyme solution from serum-free culture medium of human gastric cancer cell MKN45

Human gastric cancer cell MKN45 was cultured in a serum-free medium (RPMI1640 medium:Ham's F-12 medium=1:1) containing sodium selenite and canamycin, at 37°C in 5% CO<sub>2</sub>. The resulting serum-free culture medium (100  $\ell$ ) was concentrated to 2  $\ell$  by ultrafiltration. The buffer was changed to a Tris-HCl buffer containing 5 mM 2-mercaptoethanol and 0.1% CHAPS [3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate], pH 7.5, to give a crude enzyme solution. This crude enzyme solution was subjected to column chromatography using Q-sepharose, GDP-hexanolamine-sepharose, (GlcNAc $\beta$ 1-2Man $\alpha$ 1-6)(GlcNAc $\beta$ 1-2Man $\alpha$ 1-3)Man $\beta$ 1-4GlcNAc $\beta$ 1-4GlcNAc-asparagine-sepharose and the like to collect active fractions, from which the human  $\alpha$ 1-6 fucosyltransferase of the present invention could be purified.

(2) Preparation of enzyme

The crude enzyme solution obtained in (1) above was subjected to the following purification steps. That is, the solution was applied to a Q-sepharose column equilibrated with Tris-HCl buffer containing 5 mM 2-mercaptoethanol and 0.1% CHAPS, pH 7.5. The column was washed with a 5-fold amount of the same buffer and the active fractions eluted with the same buffer containing 0.1 M NaCl were collected. The active fractions were concentrated using an ultrafiltration membrane and the buffer was changed to Tris-HCl buffer containing 5 mM 2-mercaptoethanol and 0.7% CHAPS, pH 7.5, after which the fractions were applied to GDP-hexanolamine-sepharose column equilibrated with the same buffer. The elution was performed by the linear gradient of NaCl from 0 M to 0.5 M.

The active fractions from 0.15 M to 0.3 M were collected and concentrated using an ultrafiltration membrane. After desalting, the

fractions were applied to a (GlcNAc $\beta$ 1-2Man $\alpha$ 1-6)(GlcNAc $\beta$ 1-2Man $\alpha$ 1-3)Man $\beta$ 1-4GlcNAc $\beta$ 1-4GlcNAc-asparagine-sepharose column equilibrated with Tris-HCl buffer containing 5 mM 2-mercaptoethanol and 0.7% CHAPS, pH 7.5. The elution was performed by the linear gradient of NaCl from 0 M to 0.5 M.

The active fractions from 0.2 M to 0.5 M were collected and concentrated using an ultrafiltration membrane. Desalting gave human  $\alpha$ 1-6 fucosyltransferase.

The thus-obtained human  $\alpha$ 1-6 fucosyltransferase fractions showed a single band at a molecular weight of about 60,000 by SDS-polyacrylamide gel electrophoresis. No other activities, such as those of transferase and glycosidase, were found and this purified enzyme was sufficiently usable as a reagent for sugar chain studies.

The optimum pH (determined by changing the pH of buffer) of the enzyme of the present invention is shown in Fig. 4. The enzyme showed high activity at around pH 7.0-7.5. In this graph, the black circle shows the case when MES buffer was used and white circle shows the case when Tris-HCl buffer was used.

The pH stability of the enzyme of the present invention was examined in the same manner. Fig. 5 shows residual activity after treating the enzyme in each buffer at each pH, 4°C for 5 hours. The enzyme was comparatively stable at about pH 4-10, and particularly stable at pH 5-9. In this graph, the black triangle shows the case when acetate buffer was used, the black circle shows the case when MES buffer was used, the white circle shows the case when Tris-HCl buffer was used, and the white triangle shows the case when sodium hydrogencarbonate buffer was used.

As shown in Fig. 6, the optimum temperature of the enzyme of the present invention was found to be at about 37°C and the enzyme was considered to retain sufficient activity in the range of 20-40°C. The frozen product was stable at -20°C for at least several months.

The enzyme showed sufficient activity in the absence of divalent metal ion. Inasmuch as it showed sufficient activity even in the

presence of 5 mM EDTA, which is a chelating agent, it is concluded that the enzyme does not require a divalent metal ion.

#### Example 8

Determination of amino acid sequence of human  $\alpha$ 1-6 fucosyltransferase

Purified human  $\alpha$ 1-6 fucosyltransferase (1  $\mu$ g) was subjected to SDS-polyacrylamide gel electrophoresis, after which the protein was transferred to PVDF membrane (Millipore) by electroblotting. The PVDF membrane was stained with Coomassie Brilliant Blue G250, and a single band of  $\alpha$ 1-6 fucosyltransferase was detected at about 60 kDa. Then, the PVDF membrane containing said band was cut out, and, after destaining with 50% methanol, subjected to Biosystem 473A protein sequencer (Applied Biosystems) to determine amino terminal amino acid sequence of human  $\alpha$ 1-6 fucosyltransferase. The amino acid sequence determined is depicted in Sequence Listing, SEQ ID NO:11.

#### Example 9

Determination of partial amino acid sequence of human  $\alpha$ 1-6 fucosyltransferase

Purified human  $\alpha$ 1-6 fucosyltransferase (5  $\mu$ g) was mixed with lysine endopeptidase and subjected to SDS-polyacrylamide gel electrophoresis, after which the peptide fragments were transferred to PVDF membrane (Millipore) by electroblotting. The PVDF membrane was stained with Coomassie Brilliant Blue G250, and several bands containing peptide fragments, inclusive of two main bands, were detected. Then, the PVDF membrane containing each main band was cut out and destained with 50% methanol. Said membrane was subjected to Biosystem 473A protein sequencer (Applied Biosystems) to determine the internal partial amino acid sequence of human  $\alpha$ 1-6 fucosyltransferase. The determined amino acid sequences are depicted in Sequence Listing, SEQ ID NO:12 and SEQ ID NO:13.

#### Example 10

Preparation of probe DNA by PCR

Mixed primers shown by SEQ ID NO:14 and SEQ ID NO:15 were synthesized from the amino acid sequences obtained in Example 9. The

mixed primer shown in SEQ ID NO:14 was used as a sense primer, and the mixed primer shown in SEQ ID NO:15 was used as an antisense primer for PCR. To be specific, 36 cycles of PCR were performed wherein PCR at 94°C (30 sec), 46°C (30 sec) and 72°C (1.5 min) using 2 µg of human-derived cDNA, 25 pmole of sense primer (mixed primer shown in SEQ ID NO: 14), 25 pmole of antisense primer (mixed primer shown in SEQ ID NO:15) and a reaction mixture (50 µl) of 50 mM potassium chloride-10 mM Tris-HCl buffer (pH 8.3)-1.5 mM magnesium chloride-0.001% gelatin-200 µM dNTP, containing 2.5 units of Taq DNA polymerase, was one cycle.

The reaction mixture (10 µl) after PCR was subjected to 2.0% agarose gel electrophoresis to confirm the PCR reaction product DNA fragments. As a result, about 200 bp DNA fragment was confirmed by agarose gel electrophoresis.

This DNA fragment was subcloned into plasmid pT7BLUEt-Vector (Novagen) and the nucleotide sequence was confirmed. As a result, the DNA fragment was found to encode the amino acid sequence depicted in Sequence Listing, SEQ ID NO:12 and SEQ ID NO:13, whereby the DNA fragment was confirmed to be a part of  $\alpha$ 1-6 fucosyltransferase gene.

#### Example 11

Isolation of human  $\alpha$ 1-6 fucosyltransferase gene

The DNA fragment obtained in Example 10 was labeled with [ $\alpha$ -<sup>32</sup>P]dCTP (3000 Ci/mmol, Amersham) and used as a probe to screen clones containing cDNA encoding human  $\alpha$ 1-6 fucosyltransferase, from human gastric cancer cell MKN45-derived  $\lambda$  ZAP cDNA library by plaque hybridization method. As a result of screening of about 2,000,000 plaques, 8 positive clones c1 to c8 were obtained. Said clones c1 to c7 were postulated to contain a full length  $\alpha$ 1-6 fucosyltransferase gene in view of the restriction enzyme cleavage site and their length. The nucleotide sequences of c1 and c2 were determined, as a result of which a nucleotide sequence depicted in SEQ ID NO:9 was obtained.

#### Example 12

Expression of human  $\alpha$ 1-6 fucosyltransferase

The coding region of human  $\alpha$ 1-6 fucosyltransferase gene was

subcloned into expression vector pSVK3 from clones containing cDNA encoding the human  $\alpha$ 1-6 fucosyltransferase obtained in Example 11. An expression vector containing said  $\alpha$ 1-6 fucosyltransferase gene was introduced into COS-1 cells. After 48 hours of incubation, culture cells were collected and disrupted. The enzyme activity of  $\alpha$ 1-6 fucosyltransferase in the obtained lysate was determined. As a control, the enzyme activity of  $\alpha$ 1-6 fucosyltransferase in the lysate of COS-1 cells, into which mock pSVK3 had been introduced, was determined. As a result, the control hardly showed activity, whereas COS-cells, into which the expression vector containing said  $\alpha$ 1-6 fucosyltransferase gene had been introduced, showed a high activity of 2130 nmole/h/mg protein.

#### Industrial Applicability

The porcine  $\alpha$ 1-6 fucosyltransferase of the present invention differs significantly from known human  $\alpha$ 1-6 fucosyltransferase in physico-chemical properties, and shows activity under optimum reaction conditions which are closer to the physiological conditions.

The  $\alpha$ 1-6 fucosyltransferase derived from human also shows physico-chemical properties markedly different from those of known human  $\alpha$ 1-6 fucosyltransferase, showing activity under optimum reaction conditions which are closer to the physiological conditions. Hence, the present invention enables development of glyco-technology inclusive of modification and synthesis of sugar chain, and of a method for diagnosis of diseases, such as cancer, which includes the use of an antibody specific for the enzyme of the present invention or the gene thereof.